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Involvement of Superoxide Dismutase in Spore Coat Assembly in *Bacillus subtilis*

ADRIANO O. HENRIQUES,† LAWRENCE R. MELSEN, AND CHARLES P. MORAN, JR.*

Department of Microbiology and Immunology, School of Medicine, Emory University, Atlanta, Georgia 30322

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Endospores of *Bacillus subtilis* are enclosed in a proteinaceous coat which can be differentiated into a thick, striated outer layer and a thinner, lamellar inner layer. We found that the N-terminal sequence of a 25-kDa protein present in a preparation of spore coat proteins matched that of the Mn-dependent superoxide dismutase (SOD) encoded by the *sodA* locus. *sodA* is transcribed throughout the growth and sporulation of a wild-type strain and is responsible for the SOD activity detected in total cell extracts prepared from *B. subtilis*. Disruption of the *sodA* locus produced a mutant that lacked any detectable SOD activity during vegetative growth and sporulation. The *sodA* mutant was not impaired in the ability to form heat- or lysozyme-resistant spores. However, examination of the coat layers of *sodA* mutant spores revealed increased extractability of the tyrosine-rich outer coat protein CotG. We showed that this condition was not accompanied by augmented transcription of the *cotG* gene in sporulating cells of the *sodA* mutant. We conclude that SodA is required for the assembly of CotG into the insoluble matrix of the spore and suggest that CotG is covalently cross-linked into the insoluble matrix by an oxidative reaction dependent on SodA. Ultrastructural analysis revealed that the inner coat formed by a *sodA* mutant was incomplete. Moreover, the outer coat lacked the characteristic striated appearance of wild-type spores, a pattern that was accentuated in a *cotG* mutant. These observations suggest that the SodA-dependent formation of the insoluble matrix containing CotG is largely responsible for the striated appearance of this coat layer.

An important determinant of the resistance, longevity, and germination properties of *Bacillus subtilis* endospores is a proteinaceous structure known as the coat. The coat is assembled from a heterogeneous (in both size and amino acid composition) group of over 2 dozen polypeptides and in its final state is differentiated into a lamella-like inner layer and a striated, electron-dense outer layer (1, 11, 18, 50). Biogenesis of the spore coat is the result of a complex process of macromolecular assembly that is controlled at different levels. It involves intricate genetic regulation, with the sequential participation of at least four mother cell-specific transcription factors in the order σ^E, SpoIIID, σ^K, and GerE (31, 46). The transcriptional control guarantees that the production of coat structural components, as well as the morphogenetic proteins that guide their assembly, occurs in the mother cell chamber of the sporulating cell in a defined temporal order. However, assembly of the inner or outer coat layers does not closely reflect the order of transcription of coat structural genes (*cot*) but rather is largely dependent on a topological plan that is laid down early in the process and requires the products of three morphogenetic genes, *spoIVA*, *spoVID*, and *cotE* (3, 40, 45, 50). Mutations in these genes affect the assembly of many coat proteins around the forespore but, at least to some extent, do not interfere with the interactions among specific components. Coat structural proteins of *spoIVA* and *spoVID* mutants can still associate to form long swirls of coat material in the mother cell cytoplasm (3, 40, 45). Evidently, assembly of the spore coat involves interactions among individual coat polypeptides and mechanisms that promote interactions of higher-order building blocks.

The mechanisms enforcing these interactions are poorly understood, but the available evidence points to proteolysis and reversible or irreversible protein cross-linking (2, 8, 24, 25, 48). Inter- or intramolecular cross-linking is likely to be an important determinant of the spore coat functional architecture, since about 30% of the total coat protein is confined in a fraction that is refractory to extraction under reducing conditions and to electrophoretic analysis (37, 48). Recently, (ε-γ)-glutamyl-lysyl isopeptide bonds were detected in spores and purified coat material (25). A transglutaminase activity was subsequently purified, and the corresponding gene was cloned and characterized (24). (ε-γ)-Glutamyl-lysil isopeptide bonds are known to be present in other biological structures such as the eye lens crystallin and keratins (16, 18, 47). In other systems, dityrosine cross-links are generated by the activity of peroxidase with H_2O_2. Formation of *o*,*o*-dityrosine bonds is known to take place in the hardening of the nematode cuticle, the insect egg chorion, and the sea urchin fertilization membrane (13, 27, 43). Dityrosine bonds are also important in elicitor- and wound-induced oxidative cross-linking of plant cell wall proteins (6, 26). In all cases, cross-linking of structural proteins results in the insolubilization of specific components and confers a high degree of chemical and mechanical resistance on the final structure (16, 43, 47). Because purified coat material has a high tyrosine content, Pandey and Aronson (37) proposed that *o*,*o*-dityrosine formation could be an important mechanism in coat assembly. However, the demonstration of dityrosine cross-links in coat material has been difficult (15). In addition, the putative peroxidase(s) has not been found, nor has a system for the generation of H_2O_2.

In this report, we provide evidence reinforcing the view that oxidative cross-linking is an important mechanism in spore coat assembly. We found that a SodA mutant lacking an Mn-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, School of Medicine, Emory University, 3001 Rollins Research Center, Atlanta, GA 30322. Phone: (404) 727–5969. Fax: (404) 727-3659; E-mail: Moran@microbio.emory.edu.

† Present address: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2780 Oeiras Codex, Portugal.

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or phenotype	Source or reference
MB24	*trpC2 metC3* Spo$^+$	Laboratory stock; P. Piggot
ZB307	SPβc2del2::Tn*917*::pSK2Δ10	Laboratory stock; P. Zuber
AH394	*trpC2 metC3* SPβ*lacZ*	Laboratory stock
AH763	*trpC2 metC3* SPβ*cotE-lacZ*	This work
AH1490	*trpC2 metC3 sodA*::pAH402	This work
AH1494	ZB307 SPβ*cotG-lacZ*	This work
AH1495	*trpC2 metC3* SPβ*cotG-lacZ*	This work
AH1496	*trpC2 metC3 sodA*::pAH402 SPβ*cotG-lacZ*	This work
AH1497	*trpC2 metC3 cotG*::*cat*	This work
AH1498	*trpC2 metC3 sodA*::pAH402 *cotG*::*cat*	This work
AH1499	*trpC2 metC3 yqgE*::pAH406	This work
AH1510	*trpC2 metC3* Δ*amyE*::*sodA-gusA*	This work
AH1511	*trpC2 metC3 sodA*::pAH402 SPβ*cotE-lacZ*	This work
AH1517	ZB307 SPβ*cotH-lacZ*	This work
AH1518	*trpC2 metC3* SPβ*cotH-lacZ*	This work
AH1519	*trpC2 metC3 sodA*::pAH402 SPβ*cotH-lacZ*	This work
AH64	*trpC2 metC3* Δ*cotE*::*cat*	Laboratory stock; 19
AH94	*trpC2 metC3 gerE36*	Laboratory stock; 18

type superoxide dismutase (SOD) produces spores with altered coat layers. We propose that SodA is required to fix at least one major coat structural protein, CotG, into a structure from which it is not easily extracted. This process has dramatic consequences for the architecture of the coat.

MATERIALS AND METHODS

Bacterial strains and general methods. With the exception of ZB307 (51), all of the *B. subtilis* strains utilized in this study are congenic derivatives of Spo$^+$ strain MB24 (Table 1). *Escherichia coli* DH5α (Bethesda Research Laboratories) was used for routine molecular cloning procedures. Luria-Bertani medium was used for the routine growth of *E. coli* or *B. subtilis*. Difco sporulation medium was used for sporulation of *B. subtilis* (35). The extent of sporulation was measured by the titer of heat, chloroform, or lysozyme CFU per milliliter at 18 h after the onset of sporulation (18, 19). All of the other general techniques used were described previously (18, 19).

Extraction and analysis of spore coat proteins. Coat proteins were extracted from Renografin-purified spores as described before (18, 19). Their resolution was accomplished by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE). Electrotransfer of polypeptides from SDS-PAGE gels to polyvinylidene difluoride membranes and N-terminal sequence analysis were done as described before (42).

Cloning of a *sodA* fragment and disruption of the corresponding chromosomal locus. The N-terminal sequence (MAYELPELPY) of a polypeptide of about 25 kDa associated with the coat layers of *cotE* insertional mutant AH64 (19) matched that of several bacterial Mn-dependent SODs. We synthesized degenerate oligonucleotides corresponding to the N-terminal sequence (OM86) and to an 8-amino-acid-long region near the C terminus that is highly conserved among SOD enzymes from different species (OM87) (38, 39). The sequence of oligonucleotide OM86 is 5′-ATGGCITAYGAYCTKCCKGAYCTKCCKTAYGCI-3′, and that of OM87 is 5′-IAGRTARTAIGCRTGYTCCCAIACRTC-3′, where Y represents C+T, R is A+G, K is T+G, and I is deoxyinosine (36). A similar strategy was used to clone and characterize SOD-encoding gene fragments from several gram-positive bacteria (39). OM86 is similar to *d2* of Poyart et al. (39), except that our sequence was optimized in accordance with the *B. subtilis* codon usage (44). A single PCR fragment of about 550 bp was obtained after 30 reaction cycles of 94°C for 1 min, 50°C for 1 min and 30 s, and 72°C for 2 min. The PCR fragment was purified and inserted into *Hin*cII-digested pAH250 to create plasmid pAH402. The orientation of the insert is such that its 5′ end is close to the *Pst*I site in the vector. Plasmid pAH402 was used to convert wild-type strain MB24 (Table 1) to Sp^r. Transformants were the consequence of a single reciprocal crossover event (Campbell-type mechanism) at the chromosomal region of homology, which inactivated the *sodA* locus and produced SOD null mutant strain AH1490 (Table 1).

Disruption of the *yqgE* locus. The *yqgE* locus (GenBank accession no. D84432) resides immediately downstream of *sodA* in the chromosome (29, 32). Insertional disruption of the locus was accomplished in three steps. First, we used the PCR to generate a DNA fragment internal to the *yqgE* coding sequence. The primers used were OM185 (5′-CTGTCTTCCTCTGCAGGAATGATCGG-3′), which carries a naturally occurring *Pst*I site, and OM186 (5′-ATGTAAGTGAT**G**CAT ATGGCACAAGCA-3′), in which an *Nsi*I site was created by changing an A in the original sequence to a G (in boldface). Second, the 545-bp PCR product was purified, digested with *Pst*I and *Nsi*I, and inserted at the *Pst*I site of pUS19 (4), producing pAH406. Finally, pAH406 was used to transform wild-type strain MB24 to Sp^r. A representative transformant was picked for further analysis and named AH1499 (Table 1).

Reporter gene fusions. A PCR fragment encompassing the *cotH-cotG* promoter region (33, 41) was obtained with oligonucleotides OM118 (5′-TAATTC ACGCAAGCTTTTGGATGAACA-3′) and OM119 (5′-GGGAACGATAAG**C** TTTTTTGTGTGTGC-3′). OM118 anneals to position 38 in the *cotG* sequence (accession no. U14964) (41). The base at position 51, a T in the original sequence of *cotG*, was deleted to generate a *Hin*dIII site. OM119 anneals to position 707 in the complementary strand and incorporates an A-to-C change (in boldface), a change that also introduces an *Hin*dIII site. The 669-bp PCR product was generated with *Pfu* polymerase, purified, digested with *Hin*dIII, and cloned into pTKlac in both orientations (23). The resulting plasmids, pAH413 and pAH417, carry fusions of the *cotG* and *cotH* promoters, respectively, to the *lacZ* gene. Strain ZB307 (SPβ^S) was then transformed to Cm^r with *Sca*I-linearized pAH413 or pAH417, causing incorporation of the *cotG*- or *cotH-lacZ* fusion into the resident heat-inducible Spβ prophage (strains AH1494 and AH1517; Table 1). The MB24 lysogens of SPβ*cotG-lacZ* and SPβ*cotH-lacZ* were named AH1495 and AH1518 (Table 1). Strain AH763 is a lysogen of SPβ*cotE-lacZ* whose origin was described in reference 19.

Oligonucleotides OM180 (5′-GAGTAAAAAG**C**TTCGGAGGAAGCAAAG C-3′; the C in boldface replaces a G in the original sequence and creates an *Hin*dIII site) and OM181 (5′-TCGCGTGTCCGCCGCCGTTGTTGCGG-3′) were designed based on the sequence of the *sodA* (*yqgD*) region of the chromosome (accession no. D84432) (29). They were used to generate a 620-bp fragment encompassing the *sodA* promoter by PCR. Digestion of the PCR product with *Hin*dIII and *Bgl*II produced a fragment that could be inserted between the *Hin*dIII and *Bam*HI sites of *amyE* integrational vector pMLK83 (22), thereby creating *sodA-gusA* plasmid pAH404 (see Fig. 1). Strain AH1510 resulted from single-copy integration of the *sodA-gusA* fusion into MB24.

Other constructions. A strain carrying a null allele of *cotG* was obtained by transforming MB24 with pMS43 (a gift from E. Ricca; 41) with selection for Cm^r. The *cotG* null mutant was named AH1497 (Table 1). The same plasmid was introduced into strain AH1490 (SOD$^-$) to create *sodA-cotG* double mutant AH1498 (Table 1).

Enzyme assays. To assay for β-galactosidase or β-glucuronidase activity, 0.3-ml samples of sporulating cultures were harvested every 30 min throughout growth and sporulation. β-Galactosidase activity was assayed as described before (19, 22) and is expressed in Miller units. The specific activity of β-glucuronidase is expressed in nanomoles of *p*-nitrophenyl-β-D-glucoside hydrolyzed per milligram (dry weight) of culture per minute (19, 22).

For detection of SOD activity on nondenaturing gels, the nitroblue tetrazolium (NBT) assay was utilized (5). Strains MB24 (SOD$^+$) and AH1490 (SOD$^-$) were grown in sporulation medium, and 10-ml samples were collected at various times. The cells were harvested by centrifugation, and the pellets were resuspended in 2 ml of lysis buffer (42) and passed twice through a French press at 19,000 lb/in^2. Approximately 30 μg of protein was loaded onto nondenaturing 12.5% polyacrylamide gels, separated under nondenaturing conditions, and subjected to the SOD assay as previously described (5).

Electron microscopy. Spores were purified on Renografin step gradients as previously described (18, 19). Usually, about 10^5 spores were fixed in 3 ml of fixative. To increase the rate and depth of penetration of the fixative, a combination of 1.25% formaldehyde, 4% paraformaldehyde, and 2% dimethyl sulfoxide in phosphate-buffered saline was employed. This is an adaptation of the concentrations in Kalt's fixative (21). Following 12 to 18 h of fixation at 4°C, the cells were further processed for electron microscopy. Staining of internal structures was enhanced with additional 1-h steps in 0.01% tannic acid, 1% osmium tetroxide, and aqueous 4% uranyl acetate at room temperature. Following dehydration in a graded ethyl alcohol series, the cells were embedded in ultralow-viscosity embedding medium (28). Postsection staining was completed with 2% alcoholic uranyl acetate and calcined lead citrate (17) for 5 min each. Observation and photography of the samples were performed with a Philips CM-10 transmission electron microscope operated at 60 kV.

RESULTS

Mn-dependent SOD is associated with the inner coat layers. Coat proteins were extracted from purified spores produced by a *cotE* deletion mutant, by treatment with SDS and dithiothreitol (DTT) as previously described (18, 19). This procedure extracts a heterogeneous protein sample from wild-type spores and a less complex one from *cotE* mutant spores. After transfer of the electrophoretically resolved proteins to a polyvinylidene difluoride membrane, we obtained the N-terminal amino acid

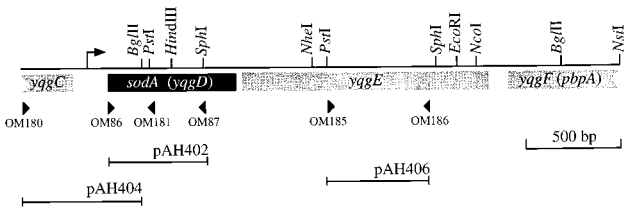

FIG. 1. The *sodA* locus of *B. subtilis*. Shown is a partial restriction map and the genetic organization of the *sodA* region of the *B. subtilis* chromosome. The horizontal lines below the genetic and physical map represent the inserts in the indicated plasmids, obtained by cloning of appropriate PCR fragments. The annealing position and the orientation of the primers used in the PCR are also indicated. The approximate location and orientation of the promoter(s) in the *sodA* regulatory region are represented by the arrow above the map.

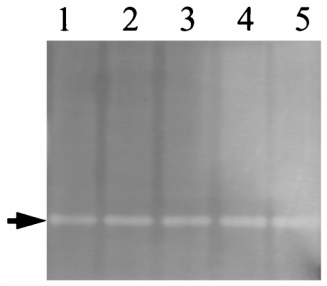

FIG. 2. Expression of *sodA* is constitutive throughout growth and sporulation. *B. subtilis* MB24 was grown in sporulation medium, and samples were taken during the logarithmic phase of growth (log) and at various times (in hours) after the initiation of sporulation (T_0). Crude extracts were prepared, and samples of protein (30 μg) were resolved on nondenaturing 12.5% polyacrylamide gels Lanes: 1, log; 2, T_0; 3, T_2; 4, T_4; 5, T_8. The gels were stained for SOD activity by the NBT method. The arrow indicates the position of achromatic bands produced by SOD activity.

sequence of a 25-kDa polypeptide and found that it matched that of several Mn-dependent SODs. We cloned a 550-bp DNA fragment internal to the *sodA* gene into an integrational plasmid, creating pAH402 (Fig. 1). Sequence analysis of the cloned insert confirmed that a fragment of a SOD-encoding gene had been cloned. The complete sequence of the gene encoding the Mn-dependent SOD from *B. subtilis* (*yqgD* or *sodA*) was later deposited in the GenBank database (accession no. D84432) (29). The *sodA* gene is predicted to encode a 25.3-kDa polypeptide, in good agreement with our initial estimation. Alignment of the complete amino acid sequence with those of other bacterial enzymes confirmed its assignment as an Mn-dependent SOD (29, 38, 39). Four conserved residues are ligands to the metal cofactor: the histidines at positions 27, 82, and 168 and the aspartate at position 164. Moreover, among the residues involved in discriminating between iron or manganese as the cofactor, the two glycines at positions 77 and 78, the histidine at position 79, the phenylalanine at position 85, the glutamine at position 149, and aspartate 150 are nearly invariant in the Mn-dependent SODs (38, 39, and data not shown). In the structurally related Fe-dependent enzymes, the corresponding positions are preferentially occupied by an alanine, a glutamine, a tyrosine, an alanine and a glycine (38).

***sodA* is expressed throughout growth and sporulation.** Because the enzyme was found to be associated with the coat layers, we wanted to know if the enzyme activity could be detected in sporulating cells at the time of coat formation or in purified spores. We prepared whole-cell lysates during the growth and sporulation of a wild-type strain and resolved about 30-μg protein samples on duplicate nondenaturing acrylamide gels which were run in parallel. The same amount of protein was loaded in each well, as shown by Coomassie staining of one of the gels (data not shown). The replica gel was then stained for SOD activity by the NBT method (see Materials and Methods). A single activity band was detected during the growth and sporulation of wild-type strain MB24 (Fig. 2). Casillas-Martinez and Setlow (7) have recently reported similar results. This SOD activity was also present in sporulating cells at the time (T_8) of coat assembly. In agreement with these results, when a fusion of the *sodA* promoter region to the *gusA* gene (Fig. 1) was introduced at the *amyE* locus, β-glucuronidase activity was detected throughout growth and sporulation (data not shown). We did not succeed in extracting the SOD activity from purified spores. One explanation for this is that our extraction conditions inactivated the enzyme. Alternatively, the enzyme is a minor component of the coat or is not extractable from the coats of wild-type spores.

Plasmid pAH402 (Fig. 1) was used to inactivate the *sodA* locus. Competent cells of wild-type strain MB24 were transformed with pAH402 to spectinomycin resistance. The transformants formed smaller colonies than those obtained in a control experiment in which the same strain was transformed to Sp^r with a plasmid generating a different insertion (data not shown). One transformant, designated AH1490, was chosen for further study. Strain AH1490 formed spores with normal levels of resistance to heat, lysozyme, or chloroform (data not shown), indicating that the spore cortex and coat were substantially intact. No SOD activity band was detected in extracts prepared from strain AH1490 at any stage of growth or sporulation (data not shown). Thus, the mutant had reduced or negligible SOD activity. From these results, and in agreement with the observations of Casillas-Martinez and Setlow (7), we conclude that the *sodA* locus is expressed constitutively throughout growth and development and that loss of detectable SOD activity does not severely impair growth or affect the resistance properties of *B. subtilis* spores.

The *yqgE* locus is not required for viability, SOD activity, or sporulation. Examination of the *sodA* region of the chromosome identified an associated downstream open reading frame designated *yqgE* in the sequence given GenBank accession no. D84432 (29). The start codon of *yqgE* is separated from the *sodA* stop codon by only 33 bp, and no possible transcription terminators or obvious promoters could be identified in the intergenic region (Fig. 1). The 429 residue YqgE protein (about 48 kDa) is predicted to have 10 to 12 hydrophobic segments, indicating a possible association with the membrane. Interestingly, genes encoding highly similar products are located downstream of SOD genes in *B. stearothermophilus* (accession no. P28754) and *B. caldotenax* (accession no. X62682). Because these observations suggested a functional linkage between *sodA* and *yqgE*, we wanted to know whether the absence of the SOD activity band in strain AH1490 was due to a polar effect on the expression of *yqgE*. For this purpose, strain AH1499 was analyzed for the presence of a SOD activity band on native gels (the strain carries a disruption of the *yqgE* locus caused by the integration of pAH406). Strain AH1499 retained SOD activity and produced wild-type levels of heat- and lysozyme-resistant spores (data not shown). We concluded that the band observed in SOD activity gels is *sodA* dependent and that expression of this activity does not require *yqgE* function. We further concluded that *yqgE* is not essential for growth of *B. subtilis*.

***sodA* mutants form spores with altered coat layers.** We hypothesized that SodA could have a role in spore coat assembly, since the enzyme was found in preparations of coat proteins and because the SOD activity was detected in whole-cell extracts at times that included the period during sporulation

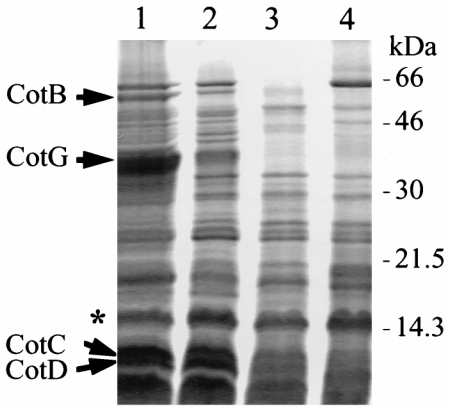

FIG. 3. Analysis of the coat layers of *sodA* mutant spores. Spore coat proteins were extracted from equivalent amounts of Renografin-purified spores produced by various strains. Lanes: 1, AH1490 (*sodA*); 2, MB24 (wild type); 3, AH64 (*cotE*); 4, AH94 (*gerE*). The samples were subjected to SDS–15% PAGE. The positions of the well-characterized coat polypeptides CotB, CotG, CotC, and CotD and molecular size markers are indicated. The extractability of the band labeled with an asterisk does not seem to vary much with the status of the coat layers (18, 19) and was used as an internal control for the amount of coat protein extracted in each case.

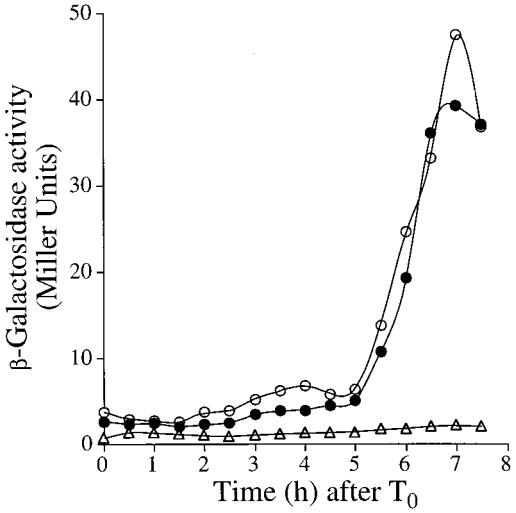

FIG. 4. Expression of *cotG-lacZ* in wild-type and *sodA* cells. Accumulation of β-galactosidase in the wild type (●) or a *sodA* mutant strain (○) lysogenic for SPβ*cotG-lacZ*. Both strains were grown in sporulation medium, and samples were taken at the indicated times throughout growth and sporulation. The end of the exponential phase of growth (T_0) is defined as the onset of sporulation. We also determined levels of β-galactosidase (△) in strain AH394 (19) carrying a promoterless *lacZ* gene.

when the coat is assembled. To examine whether SodA participates in spore coat assembly, we analyzed by gel electrophoresis the profile of SDS-DTT-extractable proteins from the coat of *sodA* mutant spores. In parallel, coat proteins were extracted from equal numbers of wild-type (strain MB24) and *gerE* (AH94) or *cotE* (AH64) mutant spores, which lack the inner or outer coat layer, respectively (30, 50). The samples of purified coat material were analyzed by SDS–15% PAGE, and examples are shown in Fig. 3. Approximately the same amount of spore suspension was used in all cases, as confirmed by the relative intensity of a band of about 15 kDa (asterisk in lane 1) whose extractability remained essentially unaffected in *cotE* and *gerE* mutants (Fig. 3, lanes 3 and 4, respectively) compared to that in wild-type spores (lane 2).

The main difference revealed in the pattern of electrophoretically resolved proteins extracted from the coats of *sodA* mutant spores is that a protein of about 36 kDa was much more abundant in the extract from the mutant spores than in that from wild-type spores (Fig. 3). Several lines of evidence indicate that the 36-kDa protein is CotG. Its apparent size corresponds to that of CotG (41), and it is missing from the coats of both a *cotE* (Fig. 3, lane 3) and a *gerE* (lane 4) mutant. CotG is an outer coat protein (and therefore dependent on CotE for assembly), and its production is controlled at the transcriptional level by the GerE protein (41). Finally, the N-terminal sequence analysis of the 36-kDa protein produced a single sequence of eight residues that matched that of CotG. Disruption of the *yqgE* locus did not affect the extractability of CotG (data not shown). Thus, the effect seen in the AH1490 mutant cannot be attributed to a polar effect of the *sodA* insertional mutation. A band corresponding to a protein of the size of CotB (9) was also more abundant in the extract from the *sodA* mutant (Fig. 3, lane 1), although this effect was not always as pronounced as in this particular gel. This band exhibited the same dependency on *cotE* reported previously (50) for the assembly of CotB into the coat (Fig. 3, lane 3).

SodA controls the assembly of CotG into coat layers. Transcription of the *cotG* gene by the σ^K form of RNA polymerase is dependent on DNA-binding protein GerE (41). GerE is a regulator that affects the transcription of several *cot* genes (49). We examined the transcription of a *cotG-lacZ* fusion integrated in single copy in the prophage SPβ in wild-type and *sodA* mutant cells. We found that the pattern of *cotG-lacZ* expression in the *sodA* mutant did not differ from that observed in wild-type cells (Fig. 4). Moreover, *cotG-lacZ* expression in the *sodA* mutant was still dependent on GerE (data not shown). We conclude that the *sodA*::pAH402 allele did not affect the transcription of *cotG*. Assembly of CotG into the outer coat layers is known to depend on CotE and CotH (33, 41). Expression of *cotE-lacZ* and *cotH-lacZ* in the *sodA* mutant did not differ from the profile obtained with wild-type cells (data not shown). Furthermore, the extractability of proteins of the size of CotE (24 kDa) or CotH (43 kDa) was not altered in coat material purified from *sodA* mutant spores (Fig. 3), suggesting that their production or assembly was unaffected. These results indicate that the increased representation of CotG in the extracts of coat proteins from spores of the *sodA* mutant is not caused by increased transcription of the *cotG* gene or by increased levels of the proteins that normally recruit CotG for assembly. It seems more likely that loss of SodA results in a change in CotG that makes it more easily extractable from the spore coat.

Ultrastructural analysis reveals that SodA and CotG are important determinants of outer coat organization. Because the *sodA* null allele affected the extraction properties of an abundant coat component, we thought the mutation would have an impact on the ultrastructural characteristics of the coat structure. We examined purified wild-type and AH1490 spores by electron microscopy. Figure 5A is an electron micrograph of a thin section of a wild-type spore. The coat, delimited by the two arrows, consisted of a lightly staining lamellar inner sublayer closely apposed to a thick, electron-dense, and multilayered outer coat (1, 18, 50). The inner coat structure usually displayed two to five lamellae, whereas the outer coat showed a characteristic pattern of striations, also two to five, depending on the section considered. In contrast, SodA mutant spores had a reduced inner coat and a highly diffuse outer coat which had lost its striated appearance (Fig. 5B). In addition, both

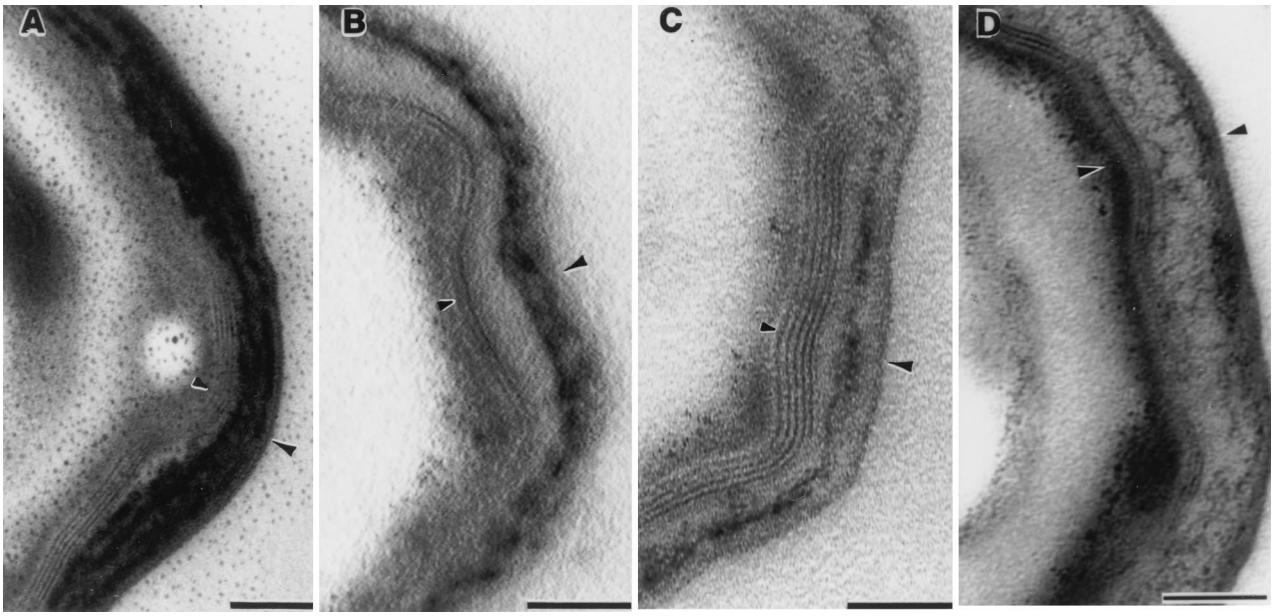

FIG. 5. Ultrastructural analysis of *sodA*, *cotG*, and *sodA*/*cotG* mutant spores. The electron micrographs show sections of spores produced by the following strains: A, MB24 (wild type); B, AH1490 (*sodA*); C, AH1497 (*cotG*); D, AH1498 (*sodA*/*cotG*). The spores were taken from cultures in sporulation medium 18 h after the onset of sporulation, purified on Renografin gradients, and processed for electron microscopy as described in Materials and Methods. In all cases, the coat layers are indicated between two arrowheads. Bars, 0.2 μm.

coat structures did not seem to associate tightly. We reasoned that if increased extractability of CotG correlated with a decrease in outer coat structural organization, then complete loss of CotG should have a much greater impact on coat morphology. As predicted, the *cotG* mutation caused a dramatic alteration in the morphology of the coat layers: spores of a *cotG* insertional mutant had an expanded outer coat that had completely lost its electron density and multilayered type of organization (Fig. 5C). Note that the outer coat appeared to be sandwiched between the inner coat lamellae and a thin layer of material at its outer edge that is slightly more electron dense than the rest of the structure. The significance of this layer is unknown. *sodA-cotG* double-mutant cells formed spores whose coat layers did not differ greatly from those of a *cotG* single mutant, suggesting that *sodA* acts mainly through *cotG* (Fig. 5D). These characteristics are consistent with a model in which CotG plays a major role in the organization of the outer coat, possibly by forming an insoluble matrix that is the basis for its characteristic multilayered appearance. We propose that SodA is used to cement CotG into this insoluble matrix.

DISCUSSION

An Mn-dependent SOD encoded by the *sodA* locus of *B. subtilis* was found to be associated with spore coat proteins. SODs catalyze the disproportionation of superoxide radicals to hydrogen peroxide and oxygen and are thought to have an important role in defending the organism against the toxic effects of oxygen (12). In agreement with the results of Casillas-Martinez and Setlow (7), we detected a single SOD activity throughout the growth and sporulation of *B. subtilis*. This activity is dependent on the *sodA* locus, since its inactivation results in cells that lack detectable SOD activity (7 and this work) and *sodA* is transcribed during growth and sporulation (this work). In confirmation of the results of Casillas-Martinez and Setlow (7), we found that *sodA* is not essential for viability in rich medium or sporulation and that inactivation of the *sodA* locus is not compensated for by the expression of other forms of the enzyme. In the bacterium *E. coli*, the *sodB* gene (encoding an Fe-dependent SOD) is expressed constitutively, whereas *sodA* encodes an inducible enzyme (12). It is unclear why in *B. subtilis* and at least two other members of the gram-positive group (14, 34) the constitutive SOD activity appears to be Mn, as opposed to Fe, dependent. The gene downstream from *sodA*, *yqgE* (29), which appears to be associated with genes encoding Mn-containing SODs in at least two other spore-forming *Bacillus* species, is also dispensable for growth or sporulation. In addition, *yqgE* is not required for the expression of *sodA* activity in crude extracts. However, the fact that it probably encodes a membrane protein and the cross-species conservation of the *sodA-yqgE* unit prompt us to speculate that the activity of SodA might be somehow coupled to a membrane-associated function.

Casillas-Martinez and Setlow (7) showed that *sodA* plays no role in the resistance of *B. subtilis* spores to oxidizing agents. Our observations suggest an alternative role for SodA during sporulation. Our results suggest that SodA participates in the assembly of the spore coat, possibly by activating the oxidative cross-linking of a specific coat structural component. Although we initially found SodA in a preparation of coat proteins, it is not known whether SodA is enriched in this fraction or whether most of the cell's SodA is located elsewhere. Since H_2O_2 produced by SodA would be diffusible, the involvement of SodA in spore coat formation does not require that SodA be a component of the coat. SodA mutant spores exhibit increased extractability of a previously characterized coat protein, CotG, an abundant spore coat component. We found that the *sodA* mutation does not affect *cotG* promoter activity. We also found that the *sodA* mutation does not affect the expression of the *cotE* and *cotH* loci (the only known requirements for CotG assembly [33, 41]) or the abundance of the corresponding products in the SDS-DTT-extractable fraction of coat proteins. The CotG protein is tyrosine rich and is organized in nine repeats of a 13-amino-acid sequence whose con-

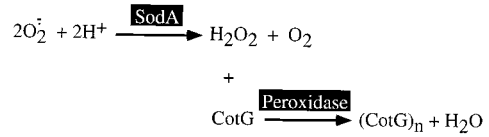

FIG. 6. Model for the role of SOD in spore coat assembly. According to the model, SodA is involved in the production of H_2O_2, which could be used by a peroxidase for the cross-linking of certain coat proteins (e.g., CotG). Therefore, CotG could partition between a soluble, easily extractable fraction and an insoluble (cross-linked or polymeric) fraction. The polymerization of CotG is represented schematically, since the exact stoichiometry of the reaction is unknown. The model does not require SodA to be a coat structural component because H_2O_2 is diffusible.

sensus is H/Y KKS Y R/C S/T H/Y KKSRS (the residues in the smaller font indicate the least-conserved positions) (41). The relatively high level of tyrosines suggests that CotG is a potential substrate for a peroxidase that catalyzes the polymerization of CotG via dityrosine cross-links. We propose that CotG can exist in two forms, a monomeric, soluble form that can be detected by analyzing a sample of purified coat material by SDS-PAGE and a polymeric, cross-linked form that is insoluble and not amenable to electrophoretic resolution. In our model, SodA promotes the oxidative cross-linking and consequent insolubilization of CotG, because it supplies the H_2O_2 substrate for the putative peroxidase. In the absence of SodA, CotG is found to be more abundant in the readily extractable soluble fraction of the spore coat (Fig. 6). Cross-linking of structural proteins via dityrosine bonds is coupled to H_2O_2 formation in two well-characterized systems, hardening of the sea urchin fertilization membrane (reviewed in reference 43) and elicitor- or wound-induced cross-linking of plant cell wall proteins (6, 26). In both cases, H_2O_2 production may be the rate-limiting step in the reaction. In the latter example, the H_2O_2-dependent cross-linking of pre-existing soluble forms of two proteins, p33 and p100, results in their disappearance from the SDS-extractable fractions of the cell wall (6, 26). Both p33 and p100 are tyrosine-rich proteins that have a highly repetitive primary structure (6, 26). These observations are strikingly analogous to our results.

A peroxidase activity has been detected during the sporulation of *B. cereus* and localized to the forespore membranes (20). In *B. subtilis*, a peroxidase activity has been proposed (37) but not detected. However, it has been proposed that CotE shares sequence similarity with heme-containing peroxidases and may have peroxidase activity (T. Diets, cited in reference 11). CotE is thought to form a ringlike structure at the interface between the inner and outer coat layers and is required for assembly of the outer coat (10, 50). Because *cotE* mutants fail to assemble the outer coat and SodA was found to be associated with coat proteins from a *cotE* mutant, SodA may, at least in part, associate with the inner coat layers. Interestingly, a nonheme catalase, the CotJC protein, is known to be a component of the inner coat layers (19, 42). This catalase could modulate the degree of SodA-dependent cross-linking, or the two enzymes may serve to protect specific cellular structures during the developmental process. The two models are not mutually exclusive.

Finally, we propose that cross-linking of CotG contributes to the multilayered pattern of outer coat striations. Support for this idea comes from the observations that the *sodA* mutation introduces distortions into this structure and that *cotG* mutant spores completely lack the normal organization and electron density of the outer coat structure. The phenotype caused by the *cotG* mutation is restricted to the outer coat layers, a finding that confirms the previous assignment of CotG as an outer coat protein on the basis of *cotE* dependency (41). Thus, both a putative peroxidase, CotE (T. Deits, cited in reference 11), and a proposed peroxidase substrate, CotG (this work), are implicated in outer coat assembly. The *cotG* mutant lacks only two of the proteins normally present in the extractable soluble fraction of the spore coat: CotG and CotB. In light of the dramatic impact of the *cotG* mutation on the electron microscopic appearance of the coat layers, one has to assume that the organization of the coat components is profoundly changed. Alternatively, proteins in the extractable soluble fraction may normally contribute very little to the electron micrographic appearance of the outer coat. In that case, the observed phenotype is due to lack of either CotG or other components of the insoluble fraction which are CotG dependent. In either case, our results point to CotG as an important organizer of the outer coat structure. We believe that this property of CotG depends, in part, on its ability to serve as a substrate for the formation of an insoluble cross-linked network upon which other coat proteins can assemble.

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